

Full Length Research Paper

Activity of red wine polyphenols on endothelial *no*-synthase (eNOS)

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Polyphenols (PPH) represent a group of chemical substances found in plants, characterized by the presence of more than one phenol unit. The largest and best studied polyphenols are the flavonoids. Polyphenols contained in red wine have beneficial effects on cardiovascular health. The aim of our research was a comparative and interactive study of four red wine polyphenols - resveratrol (R), quercetin (Q), kaempferol (K) and isorhamnetin (IH) which may have a long term properties as the increase of nitric oxide (NO) synthase expression, acting on the promoter activity when used alone or in equimolar mixtures. To determine their activity, we performed a luciferase reporter gene assay on EA.hy926 cells stably transfected with a luciferase reporter gene construct containing eNOS promoter. The Bradford assay was also performed to evaluate the cytotoxicity and the differences in cell number. The median effect equation, as an interaction analysis evaluating synergy or antagonism of the combinations was done according to the principle of mass-action law and the dose reduction index (DRI) was calculated for all mixtures. All single polyphenols activated eNOS promoter. The EC₅₀ values were in micromolar concentrations ranging from 3.44 μ M ($R^2 = 0.96$) for kaempferol to 9.89 μ M for isorhamnetin ($R^2 = 0.94$). All mixtures activated eNOS promoter, but their interactions varied from synergy (Q+R, Q+IH+K, Q+R+K and Q+R+IH+K), through additive (R+IH+K) to antagonistic interaction (R+IH, R+K, Q+IH, Q+K, IH+K and R+Q+IH). In this study, we show for the first time that red wine polyphenols activated eNOS promoter when used alone and in mixtures with different types of interactions.

Key words: Polyphenols, eNOS promoter, luciferase reporter gene assay, interaction analysis.

INTRODUCTION

Growing interest in a polyphenol rich diet has been observed in recent years. It is believed that they can protect against various diseases, e.g. cancers, cardiovascular

diseases, diabetes, and some immunological disorders (Haysteen, 2002; Xia et al., 2010). Polyphenols can counteract ROS as well as modulate signaling pathways

to enhance health outcomes in the realm of cardiovascular disorders and aging. They play a key role of Reactive Oxygen Species in Disease (Sandhya et al., 2013).

Dietary polyphenols are widely distributed in vegetables, fruits and beverages such as tea and wine. Recent studies have demonstrated that polyphenols such as resveratrol, quercetin, epigallocatechin-3-gallate and delphinidin enhance NO output to improve endothelium-dependent vascular relaxation. Moderate regular red wine consumption is associated with a reduced risk of cardiovascular diseases and is related with activation of eNOS system at different levels. Moderate ethanol intake from any type of beverage improves lipoprotein metabolism and, lowers cardiovascular mortality risk, but wine, particularly red wine with its abundant content of phenolic acids, polyphenols, and flavonoids seems to confer additional health benefits.

These include high-density lipoprotein cholesterol levels and oxidation of low-density lipoprotein (LDL) cholesterol, antioxidant activity, decreased platelet aggregation and adhesion, as well as improved endothelium-dependent vasodilatation. Many of these effects are compatible with the action of endothelium derived nitric oxide (NO) (Wallerath et al., 2003). In the development of atherosclerosis, reduced bioavailability of NO, formed by endothelial nitric oxide synthase (eNOS) precedes the appearance of visible vessel alterations (Li and Förstermann, 2000). Thus, improved NO bioavailability would be a promising step in the therapy and prevention of cardiovascular disorders (Räthel et al., 2007). As the long-term treatment of cultured endothelial cells with red wine polyphenols induces eNOS expression and causes a sustained increase in endothelial NO production. The up regulation of eNOS is probably based on synergistic mechanisms between the different polyphenolic components (Schmitt and Dirsch, 2009).

By contrast of the total contents of herbal product showing better effect than an equivalent dose of a single isolated active ingredient (Ma et al., 2009). There is an increasing awareness that analyses of single components are not always adequate to clearly assess the health benefits of natural product mixtures from dietary sources, since they involve interaction effects (Kurin et al., 2012). Interactions are generally described as being synergistic or antagonistic.

The aim of our research was an interaction study of four red wine polyphenols (Resveratrol: R, Quercetin: Q, Kaempferol: K, Isorhamnetin: IH) on eNOS promoter activation in endothelial EA.hy926 cells using median effect equations, where the effects of single compounds

and their equimolar mixtures were determined and the interactions of combinations were evaluated according to general equation for the single drug effect extended to the multiple drug effect equation for n drugs. These equations provide the theoretical basis for the combination index (CI)-isobologram equation that allows quantitative determination of drug interactions, where $CI < 1$, $= 1$, and > 1 indicate synergism, additive effect, and antagonism, respectively (Chou, 2006).

MATERIALS AND METHODS

Cell culture

The human endothelial cell line EA.hy926 (Edgell et al., 1983), stably transfected with the plasmid p-eNOS-3500-Hu-Luc-neo (Li et al., 1998) containing 3600 base pairs of the human eNOS promoter driving a luciferase reporter gene (EA.hy926-heNOS-Luc) were used for measuring the eNOS promoter activity.

Luciferase reporter gene assay

Stably transfected EA.hy926-heNOS-Luc cells were grown in Dulbecco's modified Eagle's medium without phenol red supplemented with 584 mg/ml glutamine, 100 U/ml benzyl penicillin, 100 mg/ml streptomycin (Lonza, Belgium), HAT supplement (100 μ M hypoxanthine, 0.4 μ M aminopterin, 16 μ M thymidine) (Biochrom, Germany) and 10% heat-inactivated foetal bovine serum (Gibco via Invitrogen, UK) until passage 15. For the experiments, the cells were seeded for 24 h in 96-well plates at a density of 4×10^5 cells/well and were stimulated with polyphenols - resveratrol (99% purity), quercetin (98% purity) (Sigma-Aldrich, USA), kaempferol (99% purity) and isorhamnetin (99% purity) (Carl Roth, Germany) dissolved in dimethyl sulfoxide (DMSO). Phorbol-12-myristate-13-acetate (PMA) (Alexis Biochemicals, Austria) was used as a reference (positive control) and the final DMSO concentrations in all treatment did not exceed 0.1%. Control cells were always treated with an equal volume of solvent. The concentration of single polyphenols used was 3 to 100 μ M and their equimolar combinations final mixture concentrations were 1 to 30 μ M (e.g., the 30 μ M final equimolar combination Q+R was composed of 15 μ M of R and 15 μ M of Q). After 18 h incubation with the respective compounds, the cells were washed with PBS and lysed with lysis buffer (Promega, Germany). To determine eNOS promoter activity, the luminescence generated from the luciferase activity was measured using Tecan Genios Pro (Tecan, Austria) plate reader. The values were then normalized to the protein level determined by the Bradford assay as described by Bradford with slight modifications (Bradford, 1976).

Statistical and interaction analysis

All data were obtained in three independent experiments performed in quadruplets. Data are expressed as mean $\pm$ SD. Differences between groups for statistical significance were evaluated by ANOVA with *Bonferroni post hoc test* using GraphPad Prism

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Table 1. Activation of eNOS promoter with single wine polyphenols.

Compound	EC ₅₀ (μM)	R ²
Resveratrol (R)	4.07	0.93
Quercetin (Q)	6.92	0.93
Isorhamnetin (IH)	9.89	0.94
Kaempferol (K)	3.44	0.96

software (version 5.01, GraphPad Software, Inc., La Jolla, CA, USA). P-values < 0.05 were considered significant.

The concentration of sample leading to 50% effect (EC₅₀) was calculated from the dose-effect relationship of polyphenols effect on eNOS promoter activation using GraphPad Prism software. The interaction analysis evaluating synergy or antagonism of the combinations was done according to mass - action law principle (Chou, 2006), described by Equation (1) for n-drug combination at x% inhibition, using combination index (CI) for interaction interpretation

$${}^n(CI)_x = \sum_{j=1}^n (D)_j / (D_x)_j \quad (1)$$

ⁿ (CI)_x: is the sum of the dose of n drugs that exerts x% inhibition in a combination.

In the denominator (D_x): is for D "alone" that inhibits a system x%. If CI value is =, > or < 1, an additive, synergistic or antagonistic effect is indicated.

The *dose-reduction index* (DRI) means how many-fold the dose of each drug in a synergic combination could be reduced at a given effect level compared with the doses of each drug alone. The DRI value for each corresponding drug was given for n-drug combinations:

$$(DRI)_1 = \frac{(D_x)_1}{(D)_1}; (DRI)_2 = \frac{(D_x)_2}{(D)_2} \quad (2)$$

Value of DRI > 1 indicates a favorable dose reduction, and the higher DRI value indicates the higher dose reduction for a given therapeutic effect, but does not necessarily always indicate synergism. Both CI and DRI were calculated using a median-effect analysis by CompuSyn software (version 1.0.1, ComboSyn, Inc., Paramus, NJ, USA).

RESULTS

All polyphenols activated eNOS promoter, the EC₅₀ values were in micromolar concentration ranging from 3.44 μM (R² = 0.95) for kaempferol to 9.89 μM for isorhamnetin (R² = 0.94) (Table 1). EC₅₀ (effective concentration) means the concentration (in μM) of the compound leading to the half maximal effect. EC₅₀ and R² (value quantifying the goodness of fit) were calculated using Graph Pad Prism (version 5.01, USA).

The ability of resveratrol to activate eNOS promoter described in two previous studies (Wallerath et al., 2002, 2005) and our results (Table 1) are in accordance with them.

The results of Q+R and IH+K isobolograms analysis were in concordance with the CI analysis where the CI for Q+R was 0.65 (By the definition CI < 1 indicates synergy) and CI for IH+K was 2.17 (By the definition CI > 1 indicates antagonism). We can see in Table 2, that four mixtures acted synergic (Q+R, Q+IH+K, R+Q+K and Q+R+IH+K) where the CI vary from 0.65 to 0.87. One mixture effect was nearly additive: R+IH+K with CI = 1.08 and other six mixtures were antagonistic with CI from 1.16 to 2.01.

DISCUSSION

NO is one of the main mediators of vasodilatation. Its decreased level plays a central role in endothelial dysfunction. In mammals, endothelial NO is produced by the enzyme eNOS, which converts L-arginine in the presence of O₂ and NADPH into L-citrulline and NO (Appeldoorn et al., 2009). The generation of NO plays a major role in maintaining cardiovascular homeostasis by governing blood pressure, improving endothelial function, suppressing vascular smooth muscle mitogenesis, inhibiting leukocyte adhesion and platelet aggregation.

Dietary polyphenols are widely distributed in vegetables, fruits and beverages such as tea and wine. Average total of polyphenol content measured by the Folling method is 216 mg/100 ml for red wine and 32 mg/100 ml for white wine. The content of phenols in rosé wine (82 mg/100 ml) is intermediate between that in red and white wines.

Recent studies have demonstrated that polyphenols such as resveratrol, quercetin, epigallocatechin-3-gallate and delphinidin enhance NO output to improve endothelium-dependent vascular relaxation (Xu et al., 2004).

Moderate regular red wine consumption or the consumption of red grapes, fruit, cereals, several vegetables such as red onions, chocolate, tea, and coffee with different polyphenolic composition (Bravo, 1998; Tsao, 2010) is associated with a reduced risk of cardiovascular diseases and is related with activation of eNOS system at different levels (Wallerath et al., 2005).

In this study, we investigated the influence of resveratrol, quercetin, kaempferol and isorhamnetin: Polyphenols present in red wines, on the eNOS promoter activity. Both individual substance or in their equimolar mixtures were investigated. Further, we evaluated their interactions when used in combinations.

As it was described in Materials and Methods, first of all we explored activity of single polyphenols on eNOS promoter activation in four different concentrations of polyphenols (3-10-30-100 μM) and from the dose-effect relationship the EC₅₀ values using GraphPad Prism software were determined.

As it is seen in Table 1, all polyphenols activated eNOS promoter, where the EC₅₀ values were in micromolar concentration ranging from 3.44 μM (R² = 0.96) for kaempferol to 9.89 μM for isorhamnetin (R² = 0.94).

Table 2. EC₅₀, CI, and DRI values of polyphenol mixtures at 50% effect dose level.

Polyphenol mixture	EC ₅₀ (μM)	R ²	CI	Interaction	DRI
R+Q	3.30 (1.65 : 1.65)	0.97	0.65	Synergy	2.5 : 4.2
R+IH	6.75 (3.38 : 3.38)	0.99	1.16	Slight antagonism	1.2 : 3.0
R+K	4.50 (2.27 : 2.27)	0.95	1.22	Moderate antagonism	1.8 : 1.5
Q+K	5.41 (2.71 : 2.71)	0.89	1.18	Slight antagonism	2.6 : 1.3
Q+IH	11.39 (5.74 : 5.74)	0.96	1.41	Moderate antagonism	1.2 : 1.7
IH+K	11.09 (5.54 : 5.54)	0.95	2.17	Antagonism	1.8 : 0.6
R+Q+IH	7.30 (2.43 : 2.43 : 2.43)	0.99	1.20	Slight antagonism	1.7 : 2.8 : 4.1
Q+IH+K	4.88 (1.63 : 1.63 : 1.63)	0.95	0.87	Slight synergy	4.2 : 6.1 : 2.1
R+IH+K	5.04 (1.68 : 1.68 : 1.68)	0.97	1.08	Additivity	2.4 : 5.9 : 2.0
R+Q+K	3.50 (1.16 : 1.16 : 1.16)	0.97	0.79	Moderate synergy	3.5 : 5.9 : 2.9
Q+R+IH+K	3.60 (0.91 : 0.91 : 0.91 : 0.91)	0.97	0.71	Moderate synergy	7.6 : 4.5 : 10.8 : 3.8

Polyphenols equimolar mixtures: R - resveratrol; Q - quercetin, IH - isorhamnetin, K - kaempferol. EC₅₀ (effective concentration) means the concentration (in μM) of the compound leading to the half maximal effect. EC₅₀ and R² (value quantifying the goodness of fit) were calculated using Graph Pad Prism (version 5.01., USA). CI - combination index, based on the mass-action law is quantifying drug interaction in terms of synergy (CI <1), additivity (CI =1) or antagonism (CI >1). DRI represents the order of magnitude (fold) of dose reduction that is allowed in combination for a given degree of effect as compared with the dose of each drug alone. CI and DRI were calculated using CompuSyn software (version 1.0.1, USA). Interactions are determined according to Chou (2006).

Wallerath et al. (2005) demonstrated that quercetin has no effect on eNOS promoter activity up to 33 μM. However, in our experiment, we found out that quercetin activated eNOS promoter (EC₅₀ 6.92 μM; R² = 0.93). We also demonstrated that quercetin activates eNOS promoter not only alone but also in mixtures with other red wine polyphenols (Tables 1 and 2).

It is known that kaempferol significantly induces NO production in endothelial cells (Chen et al., 2010) and isorhamnetin has shown inhibitory effect on ox-LDL induced eNOS down regulation (Bao and Lou, 2006), but for the first time we described that kaempferol and isorhamnetin activate eNOS promoter (Table 1).

Resveratrol has been shown to enhance the expression of eNOS modulate the deacetylation of eNOS and increase the plasma NO levels (Wang et al., 2012). The ability of resveratrol to activate eNOS promoter was described in the two previous studies (Wallerath et al., 2002, 2005) and our results (Table 1) are in accordance with them.

It is also known that resveratrol has protective effects on multi-targets related to cardiovascular diseases. It seems that a drug targeting multiple points may exhibit better therapeutic efficacy than one target blocking or activating in complex conditions. Common disorders such as cardiovascular diseases tend to result from multiple molecular abnormalities (Wang et al., 2012), thus multi-targeting drugs or combinations of drugs seem to bring much more efficiency into therapy or prevention. We prepared binary, tertiary and quaternary mixtures of tested polyphenols in four concentrations (1-3-10-30 μM), where the contribution of each part was always equimolar (e.g. the 30 μM final equimolar combination Q+R was composed of 15 μM of R and 15 μM of Q) and gave the same final molar concentration of the mixture as the

single compound samples. As it is shown in Table 2, EC₅₀ values of polyphenols mixtures ranged from 3.31 μM (R² = 0.97) for R+Q to 11.09 μM (R² = 0.95) for IH+K.

When we used the combination index analysis based on the mass-action law for quantifying drug interactions, we were able to determine not only binary mixtures interactions but also perform n>2-drug combinations interactions analysis.

The interaction studies, which determine synergy or antagonism of substances, are relatively well known for at least three decades amongst antioxidants. Nevertheless, there is no information about red wine polyphenols interactions related to eNOS pathway.

Räthel et al. (2007) investigated apart from resveratrol also red wine polyphenol extracts (RWPE) from 180 wine types. Using luciferase reporter gene expression (Kurin et al., 2013) as an indicator for eNOS promoter activity they found out that all RWPE under investigation increased eNOS promoter activity, but the biological activity was dependent on an individual polyphenol pattern.

When they compared the RWPE results with resveratrol, they discovered that resveratrol mimics the effects of RWPE at concentrations higher than that calculated to be present in analyzed wines and thus, resveratrol alone does not account for the observed effects of RWPE. Thus, synergy with other compounds in red wine is suggested (Räthel et al., 2007). Chan et al. have shown that the effects of ethanol on NO production and inducible nitric oxide synthase (iNOS) gene expression in murine macrophage cells (RAW 264.7) was synergistically increased when combined with quercetin and resveratrol in reducing NO production by both scavenging NO and reducing iNOS gene expression (Chan et al., 2000). We found out that quercetin with resveratrol act synergistically in eNOS promoter activation (Table 2). This is in

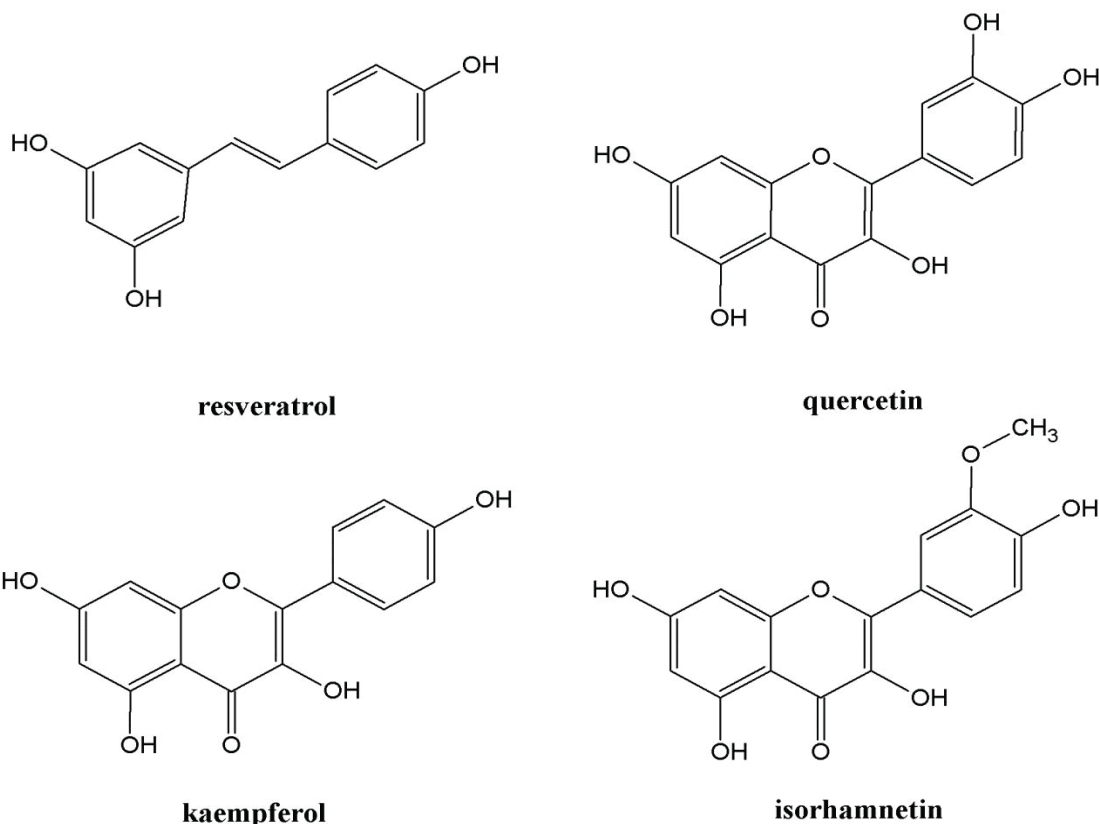


Figure 1. Structures of resveratrol (R), quercetin (Q), kaempferol (K) and isorhamnetin (IH).

accordance with the results where resveratrol and quercetin, synergistically inhibited vascular smooth muscle cell proliferation when used in a mixture (Kurin et al., 2012).

Besides CI we determined the DRI as well. DRI represents the order of magnitude (fold) of dose reduction that is allowed in combination for a given degree of effect as compared with the dose of each drug alone, or in other words it indicates to what extent the concentration of drug can be reduced in a mixture in order to achieve a given effect level compared with a single drug treatment. DRI values higher than 1 are desirable, but they do not necessarily indicate synergy. As seen in Table 2, in the Q+R mixture are DRI values 2.5 for quercetin and 4.2 for resveratrol, what means that in Q+R mixture we needed 2.5 times lower dose of quercetin and 4.2 times lower dose of resveratrol to achieve the same effect that would be reached by the single compound treatment.

Despite we are not able to explain the inner mechanism of interactions among tested red wine polyphenols in eNOS promoter activation, we've take into account that as the eNOS promoter activation involves multiple processes, the interference with multiple different targets is needed. Herbal drugs as complexes of substances or prepared mixtures of natural compounds open the

possibility of novel multicomponent treatment or prevention approach development through synergistic interactions, which could impact multiple targets simultaneously, thus being better suitable for controlling complex diseases or biochemical pathways such as eNOS (Zimmermann et al., 2007).

In a small experimental model, we have shown that red wine polyphenols when used in mixtures are needed in a smaller amount and reach many times higher effects that is single molecule able to. "French paradox" until today has not been explained by a single effective molecule, our work suggests that the positive effects of red wine on cardiovascular system should be explained by the synergy of polyphenols mixtures present in red wine, thus despite their low concentration, their effects could be given by their cooperation in multiple system.

Conclusion

Resveratrol, quercetin, kaempferol and isorhamnetin, the substances present in red wine, can activate eNOS promoter when used alone or in equimolar mixtures Figure 1. The interaction study of red wine polyphenols indicated that in eNOS promoter activation, the final effects of mixtures vary from synergistic to antagonistic.

Currently, the mechanism of their interaction is not known. However, when they are used together in a quaternary mixture, the final effect is synergic. In summary, the presented data support the idea that red wine contains unique polyphenolic constituents that may increase eNOS expression and thus endothelial NO output. More work is needed, however, to determine the bioavailability and pharmacokinetics of polyphenols and to identify metabolites of red wine components that may mediate red wine activity.

Conflict of interest

The authors declare no conflict of interest.

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